Cultured Neuron Probe

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General Introduction

Our aim is to develop a cultured neuron probe. This consists of a silicon structure into which individual dissociated neurons can be placed, and which can be inserted into an intact nervous system. Furthermore, within the structure each neuron is in close proximity to an electrode, by means of which it can be stimulated, or its activity can be recorded, through electrical leads which connect to external electronics. It is hoped that neurons in the probe will synaptically integrate with the host nervous system, to provide a highly specific, relatively non-invasive, two-way communication channel. If this occurs, the methodology has important possibilities for neural prostheses. The goal of this project is to perform initial experiments to establish the feasibility of communication by means of a neuron probe. The tissue we have chosen in which to initially implant the probe is the rat hippocampus. If initial studies are successful, probes will be designed and implanted for communication with sensorimotor cortex.

The neuron probe we plan to fabricate will be made of micromachined silicon and will have sixteen electrodes: one conventional electrode to detect activity during placement of the probe, and fifteen within wells into which neurons will be placed. Its configuration will be similar to that of passive multielectrode probes which have been developed. It will be implanted when the cultured neurons are very young, and after a time of weeks it is hoped that they will have survived and made two-way synaptic connections. By stimulation and recording in the host and probe, we will test for the existence of such connections. An essential feature of the experiments is that the viability of the implanted neurons will be independently determined over time by stimulating them and recording their resulting action potentials.

Summary

In Vitro Studies

For studies of outgrowth into long-term hippocampal slice cultures, development of good Di-I staining procedures has continued. Although it is possible to get stron staining of newly plated cells by using sucrose solutions, this results in dye binding to the probe so that it then stains the slice indiscrimately. Studies of staining of dissociated cells are being pursued to obviate this problem.

Neurochip tests have been pursued with both SCG neurons and hippocampal pyramidal cells, and progress has been made in achieving better attachment in the wells and subsequent outgrowth. In addition, a better procedure for feeding the cultures after the first few days has resulted in long-term growth. The problem of cells climbing out of the wells is still with us, and is being studied with time lapse photography. A few initial attempts to record from the wells for very young cultures were not successful, but we believe that we are close to being able to make electrophysiological measurements.

Fabrication

Studies were made of the strength of the neuroprobes in buckling, to complement those previously made in bending. These tests are particularly relevant for successful penetration of outer brain structures, and there was some concern about weakness arising from the wells. The results indicate that there will be no problems.

In addition, production of neuroprobes continued, and the processes were refined to provide smoother probe edges.

In vivo studies

Experiments continued with septal cells, many of which are cholinergic, inserted into neuroprobes probes. Probes loaded with septal cells were transplanted into the hippocampus of adult rats with fimbria-fornix lesions,

which eliminated all normal cholinergic hippocampal fibers. Acetyl-cholinesterase (AChE) staining was done 1-2 months after transplantation. In two of the five rats, cholinesterase-stained fibers were revealed in proximity to neuron probes, indicating outgrowth from probe neurons. The fiber outgrowth was copious and extended over distances exceeding100 microns.

Many implanted probes did not exhibit outgrowth, at least partially due to a poor yield of cells attaching to wells and remaining in them. Studies aimed at improving this situation will be pursued in the coming quarter, and probes will not be implanted unless many celols remain in wells.

Cultured hippocampal slice studies

Di-I labeling of probe neurons

We would like to be able to observe the outgrowth of neuronal processes from the probe wells into a hippocampal slice maintained in culture, as described in the previous quarterly report. Thus, we have been investigating ways to stain the probe neurons with the lipophillic vital dye, Di-I, before the probe is placed in contact with the slice. If we can monitor the growth of stained neural processes into the unstained slice using fluorescence microscopy at a number of stages during the integration process, we will be able to determine which variables are important for successful integration. This approach will provide information about the dynamics of integration that can not be obtained from our *in vivo* studies.

There are two basic approaches to staining the probe neurons. They can be stained in suspension immediately after dissociation, or they can be stained after they have been inserted into the probe wells and started growing. During the last quarter we have been investigating the merits and the drawbacks of each approach.

We have found that a ten-minute exposure of plated hippocampal cells to an isoosmotic sucrose solution of 40 ug/mL Di-I causes neurons and their processes to be stained brightly for at least one week, without impairing their viability. Neurons were plated onto polylysine/laminin-coated probes, grown for one or two days, and then stained in this manner. After rinsing twice with isoosmotic sucrose, the probes were inverted onto slices, the stained neurons being in direct contact with the slice. After 3 days, many of the cells in the slices had become stained, obscuring those originally plated into the probes. In control experiments using stained probes that had no neurons, it was determined that much, if not all of the dye transfer was sue to non-specific binding to the probe substrate. Careful rinsing was ineffective at preventing this problem. An approach in which the probe itself is never exposed to a dye solution would appear to be necessary.

Dissociated hippocampal cells can be stained in suspension, although this process is more traumatic to the cells than staining after plating, because rinsing requires several steps of centrifugation and resuspension of the cells by trituration. Although a sucrose solution is ideal for staining plated neurons due to the enhanced solubility of Di-I compared to its solubility in media, the density of isoosmotic sucrose prevents pelleting of stained cell suspensions by centrifugation. Therefore, we are assaying a number of alternative staining solutions and rinsing protocols at a number of Di-I concentrations to maximize brightness of staining and cell viability, while minimizing stained non-cellular debris and precipitation of Di-I crystals. If Di-I crystals and stained debris are transferred to the probes along with the probe neurons, then we will suffer from the same problem of the probe neurons being obscured by stained cells in the slice. Our most effective staining protocol thus far is the following:

Prepare a 40 mg/mL Di-I stock in dimethylformamide.
Add 1 uL of stock to 2 mL of Neurobasal/B27 medium at 37°C (20 ug/mL final).
Add a suspension of approximately 500,000 freshly dissociated cells from an E17 rat hippocampus.
Incubate 30 min at 37°C.
Centrifuge at 100 x g for 3 min.
Carfully remove supernatant and rinse pellet twice with warm medium.
Gently triturate pellet in 0.25-0.5 mL of medium using a pasteur pipet.
Plate at the desired density on polylysine/laminin-coated dishes or probes.

Time-lapse videography of newly-plated neurons

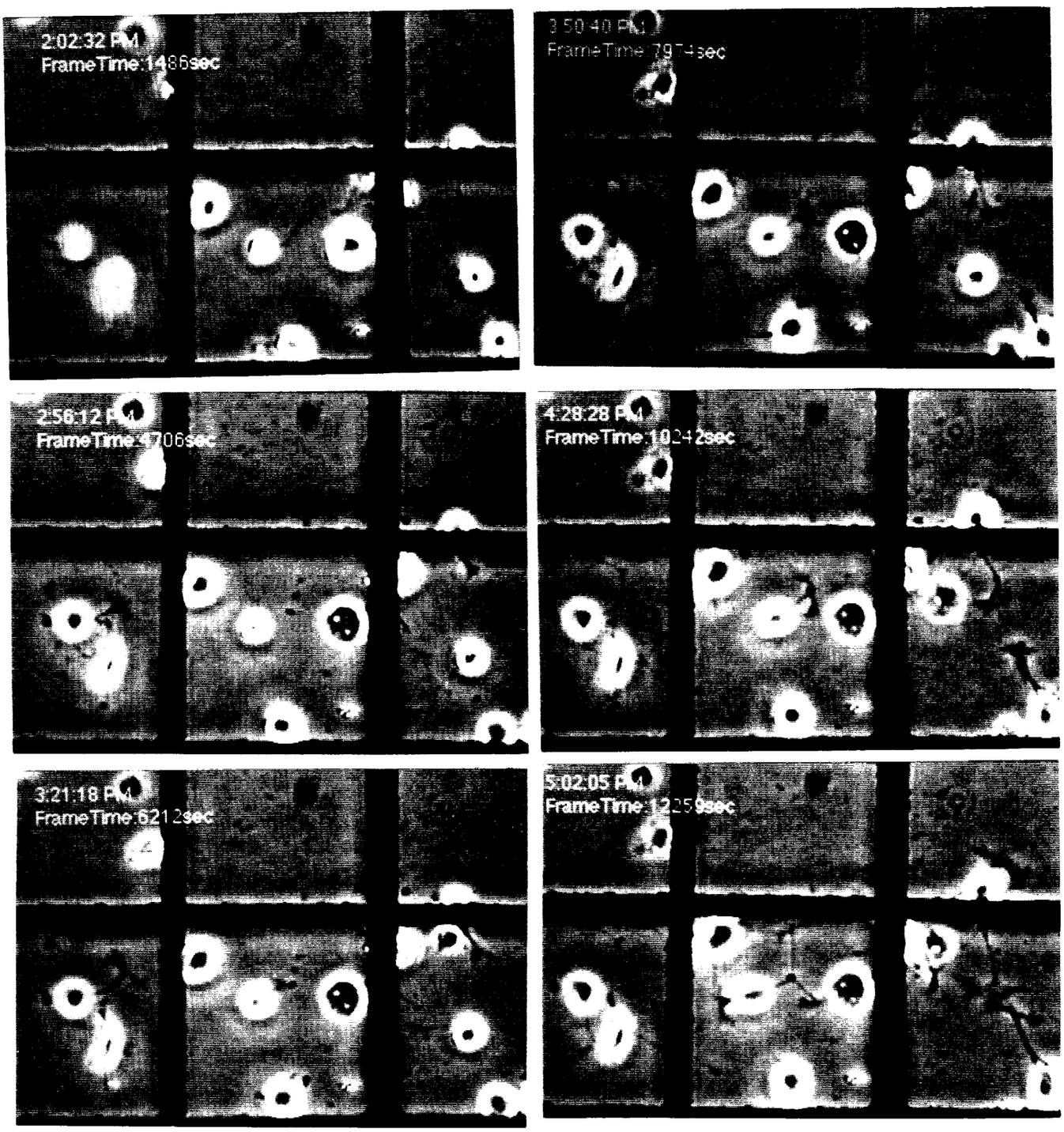
Dissociation of rat hippocampus produces a heterogeneous mixture of cells, including pyramidal neurons, interneurons, glia, blood cells, etc. For a couple of hours after dissociation, these are mostly all round and do not at all resemble their final forms after they have adhered to the substrate and established themselves in culture. When inserting cells into probe wells, we must choose a few from this heterogeneous mixture that we think will become healthy neurons. To aid the process of recognizing healthy neurons right after

dissociation, we have set up a computer-controlled time-lapse video microscope. We plate freshly dissociated cells onto polylysine/laminin-coated dishes, and record the transition from being a variety of sizes and types of round balls to being a culture of recognizable neurons and other cells. Preliminary experiments using cells from dissociated superior cervical ganglia (SCGs) from newborn rats have been immensely successful. Within three hours after dissociation, many SCG neurons are sending out long processes, as shown on the following page. The SCG cells from a P0 rat pup were dissociated and plated at 1:30 PM, and the images were taken at the times indicated in the upper-left corners. The squares of the grid are 100 um across.

SCG neurons grow about four times faster than hippocampal neurons. Thus, we must keep hippocampal cells healthy overnight on the microscope stage in order to watch them develop. We are now optimizing media in which hippocampal cells can grow in an air atmosphere. (All of our hippocampal cultures have bicarbonate-buffered media, designed for a 5% carbon dioxide atmosphere.) By plating the cells onto dishes with numbered grids, we can easily return to the same field of cells for subsequent observations of cell differentiation and growth.

High Speed Videography

The project to develop high speed CCD videography for voltage sensitive dye recording in the slices has further progressed. The initial camera electronics has been made faster, so that a digitizing rate of 1 megapixel per second is available. This still does not permit kilohertz frame rates, but was a next step in that direction. During the coming quarter construction will begin on new electronics designed to reach kilohertz frame rates for a chosen selection of pixels from the full frame.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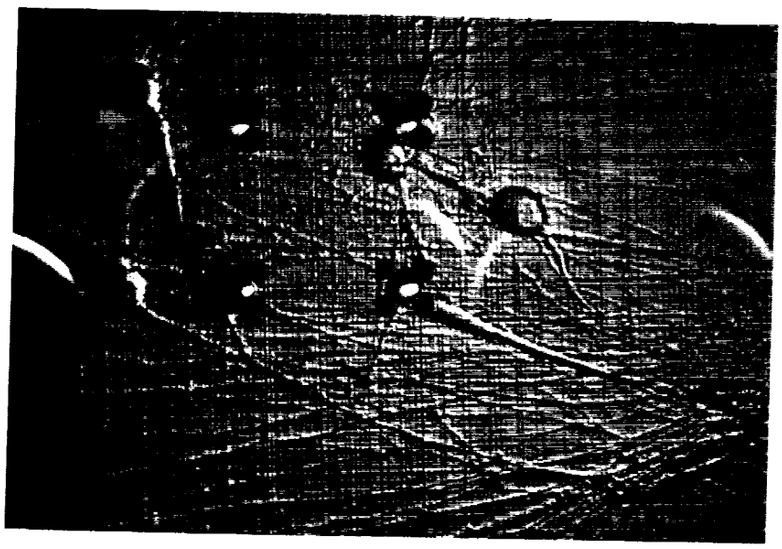
Neurochips

In the past 3 months, we have isolated and eliminated two major problems preventing neuronal outgrowth from neurochip wells: recurrent infections and shock induced by feeding. In order to load the wells on the chip, it is necessary to remove the cover of the tissue culture dish in a non-sterile environment for a period of hours. This results in a high risk of infection, even in the nominally clean environment of the lab. Therefore, we have developed a dish cover which allows observation of chip and side access for micromanipulators, while keeping the top covered. Only a small notch in the side of the dish is exposed to the air, vastly reducing the entry of airborne bacteria. This notch can easily be closed by rotating the dish cover, sealing the dish when micromanipulators are not in use.

Cultures of hippocampal and SCG neurons must be fed after four days. We had previously noted a "mid-life crisis" stage for both cell types, during which most neurons become sick-looking and many die. This crisis almost invariably occured the day after the first feeding. We altered the timing of this first feeding in hippocampal neurons to between two and seven days in culture, with the same results. Both cell types undergo a vigorous growth period during the first two weeks in culture, so it is possible that the feeding alters the medium chemistry too rapidly for the cells. (This is somewhat surprising in light of the observation that removing the cultures from the CO_2 incubator for light microscopy for times on the order of an hour during the first week does not appear to affect cell viability, even though the temperature, pH, and pCO_2 change drastically over a 15 minute time scale.)

We tested this hypothesis by feeding the cells using medium equilibrated in the incubator containing the cells. Preliminary results indicate a marked improvement in the survival rate after the first feeding using equilibrated medium. Using this method, we were able to maintain SCG neurons in neurochip wells for up to three weeks, long enough to attempt electrophysiology. Unfortunately, only one cell on each of three neurochips remained in the wells; on these particular chips, most cells escaped the wells. Below is a photograph of one of these chips. A 5 μm wide dendrite can clearly be seen emerging from the lower right corner of the lower right well.



We attempted to record somatic electrical activity at the neurochip electrodes by stimulating axons with an extracellular micropipette as a current source on two of these chips after 4 days in culture. Several factors contributed to our failure to record any such activity:

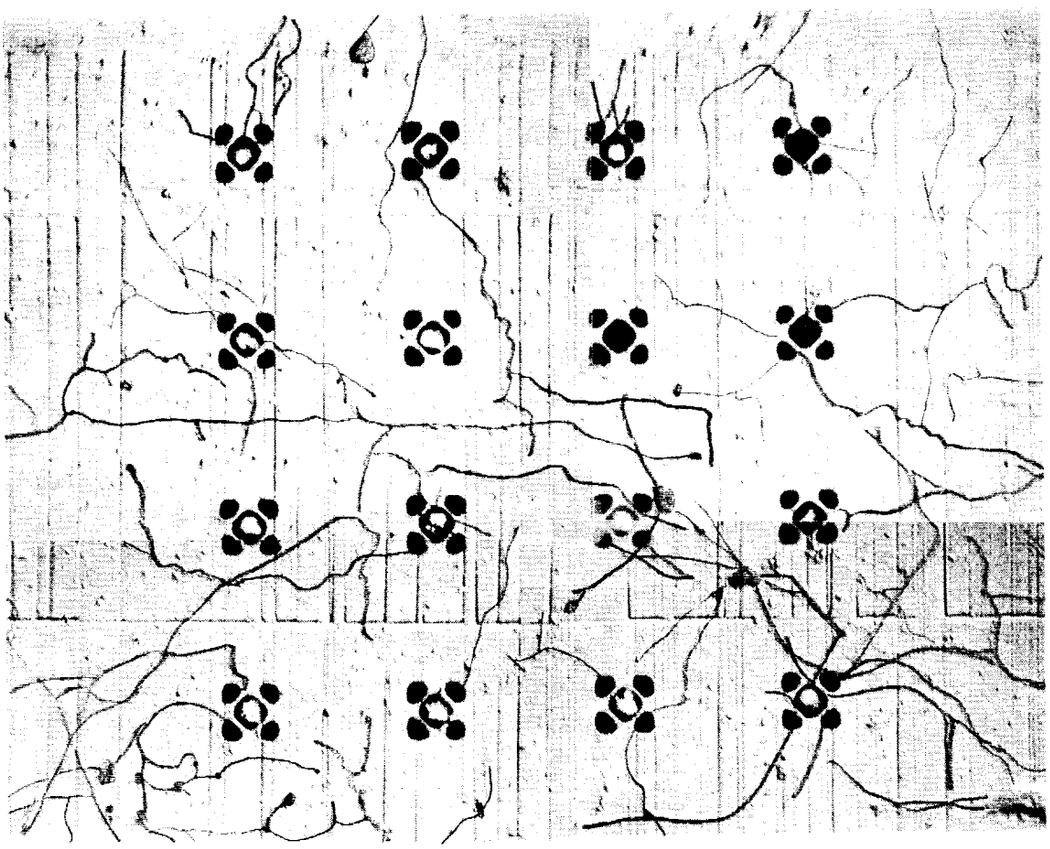
(1) Attempts to stimulate action potentials in SCG cell bodies in standard culture dishes at 5 days yielded a success rate of about 20%. Approximately 35% of healthy-looking cells could not fire AP's even with intracellular stimulation. The remaining 45% of cells would only exhibit subtheshhold excitation when stimulated extracellularly at individual axons.

(2) On some of the older chips, only a few electrodes actually work, due partially to excessive stresses placed on the membrane by the conformal coating used to support the membrane. If the SCG neurons discussed above were not in wells with good electrodes, we would not be able to see any electrical activity. We are still optimizing the chip-mounting procedure. New chips are being mounted using a glass cover slip to provide support; the first chip mounted in this way had 14 of 16 electrodes active.

(3) We are still optimizing the procedure for keeping cells in wells. Both chips had several neurons escape their wells. One possible reason for this may be that our chips are *too* clean. Poly-L-lysine and laminin both normally require 45-120 minutes to adhere to glass, but silicon appears to be less adhesive. We have found that overnight applications of adhesion factors are required for clean silicon surfaces.

We have also made progress in growing fetal rat hippocampal pyramidal cells in neurochip wells. The figure below is a photomicrograph of a neurochip

after 3 days in culture. Due to the low contrast and the difficulty in reproducing the photograph, the processes have been artificially darkened. Processes can be seen emerging from nine wells: (row, column) (1,1), (1,2), (1,3), (1,4), (2,1), (2,4), (3,2), (4,1), and (4,4). Well (4,4) contains a glial cell, as determined by the greater width and lower contrast of the processes compared to those of neurons. Unfortunately, this culture died after feeding on Day 4 in culture. However, we feel that we are very close to maintaining both SCG and hippocampal cultures long enough to successfully attempt electrophysiology.



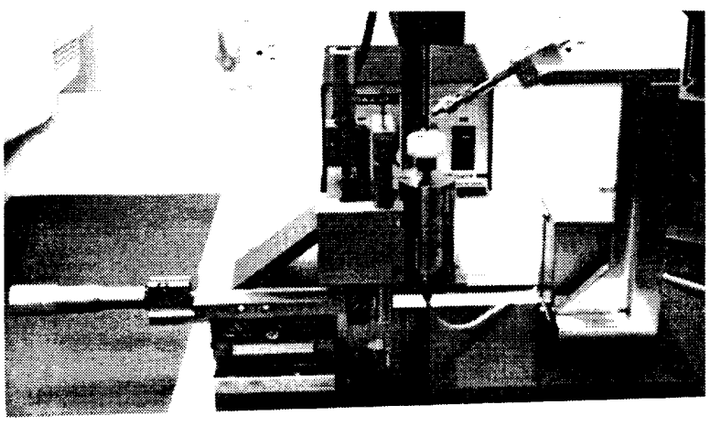
Fabrication

In the past quarter, the focus of our attention was on the mechanical testing of the probes, refining of the fabrication process, and continuing the fabrication of neurochips and neuroprobes.

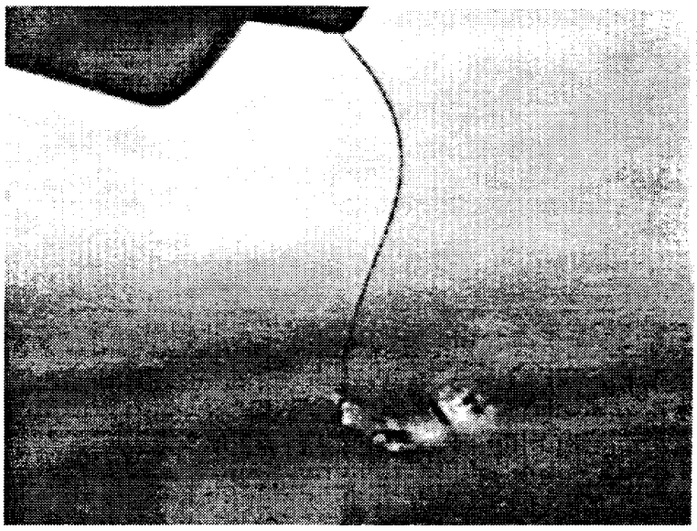
Buckling Tests

Besides the bending testing of the probe performed earlier, buckling tests of the probe have been done. The buckling of the probe is believed to be the most frequent type of probe's deformation during the insertion process. Knowing that our probes are have neuron wells which might have an influence on fragility of the probe, special attention was devoted to their mechanical testing. It has been shown that they are strong enough to withstand the insertion process.

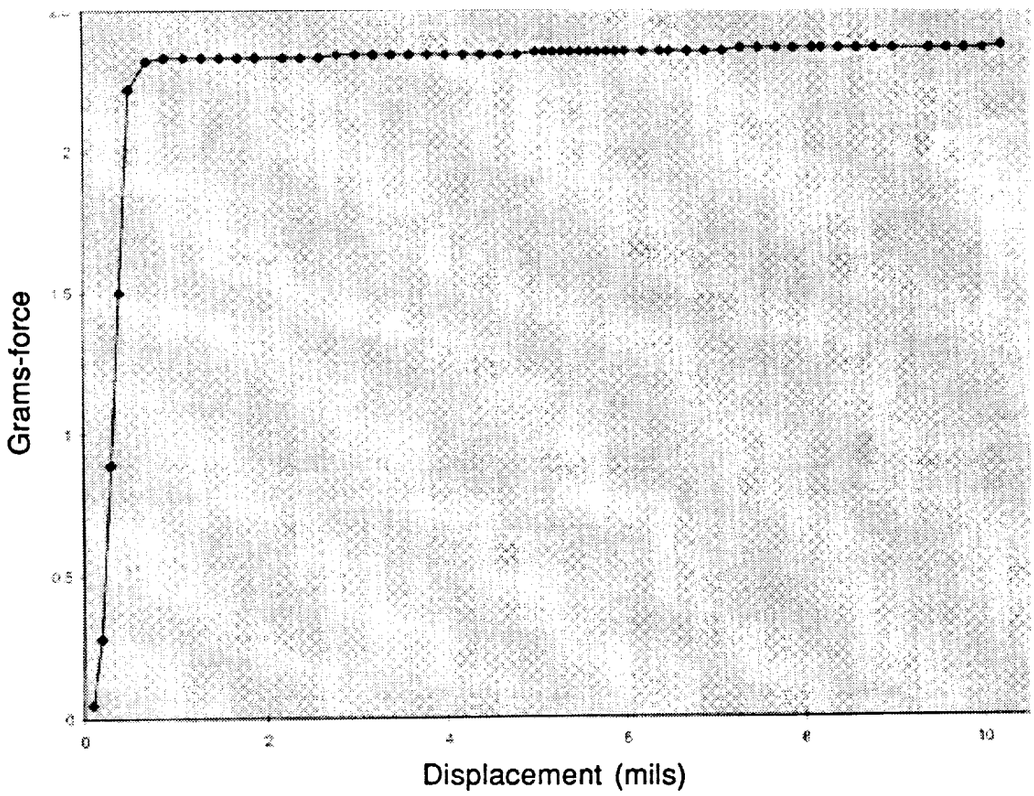
The buckling testing was performed on the same static testing setup, shown below, as the bending test that was done before. Basically, it consists of a load cell on which the probe can be mounted, and that measures the force exerted on the fixed stylus positioned above the sample. The corresponding displacement with respect to the initial position is measured by a Linear Voltage Differential Transformer (LVDT).



A probe during a typical buckling test is shown below.



A loading curve obtained using this setup is shown below, indicating that the the probe is very compliant when exposed to buckling. It was taken just before the breaking of the probe.



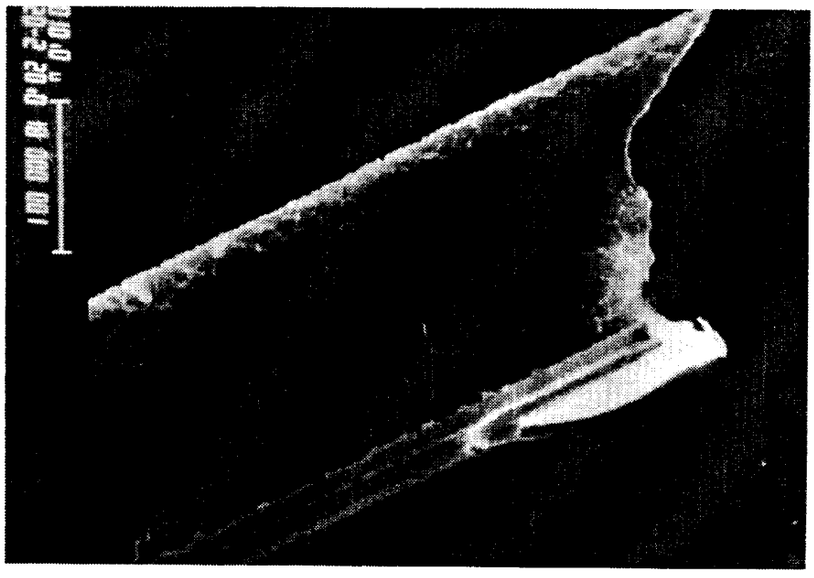
On the basis of the measurement of the probe's deflection from the vertical direction y_a, one can estimate the maximum stress that probe can endure without breaking. For a cantilever beam of thickness t, length L, width W and Young's modulus E the maximum stress on the shank occurring during buckling is

$$s_{max} = \frac{6E\,t\,y_a}{L^2}$$

For the cantilever beam the maximum stress is independent of the width of the probe. Assuming that the same is valid for a cantilever beam of non-uniform cross section (a somewhat bald assumption that yet has to be checked), one can calculate that for our probe $s_{max} = 8.7 \times 10^9$ dynes/cm^2. This is thought to be strong enough for the penetration of rat pia, arachnoid, and dura layers. It might be increased, though, by changing the probe width. Silicon substrates with small cross-sectional area have a fracture stress at least 5-6 times higher than that of bulk silicon.

Fabrication

The fabrication process for the neuroprobes has been continued and some details examined and refined. The analysis of SEM pictures of the probe's edge showed that the last step, RIE etching, leaves a very rough surface on the side walls of the probe, shown in the figure below.



However, SEM analysis of the side walls of dummy neuroprobes, where the last fabrication step is EDP etching, shows atomically smooth crystal planes. Knowing that, it has been decided that probes with electrodes are going to be immersed briefly in EDP after the RIE etching to smoothen the edges of the probe and enable insertion with the minimum damage. The success of this is illustrated in the figure below.

In Vivo Studies

Summary

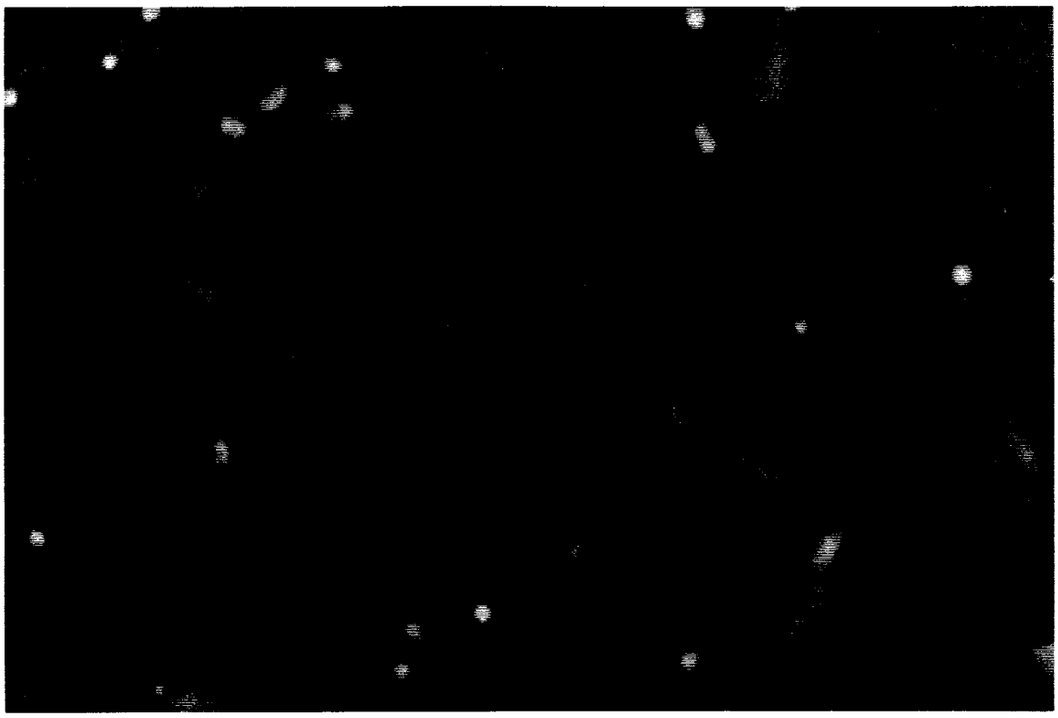
During this period we continued experiments with culturing septal cells, many of which are cholinergic, within silicon probes. Probes loaded with septal cells were transplanted into the hippocampus of adult rats with fimbria-fornix lesions, which eliminated all normal cholinergic hippocampal fibers. Acetyl-cholinesterase (AChE) staining was done 1-2 months after transplantation. In two of the five rats, cholinesterase-stained fibers were revealed in proximity to neuron probes, indicating outgrowth from probe neurons.

Results

About 600 neurons were moved into the wells of 42 probes. Approximately half of the probes had already been used in previous experiments. These probes were cleaned by the protocol described in our previous quarterly report. We have chosen 0.1% poly-L-lysine for covering the surface of the probes, with the expectation that smaller molecular weight polylysine may better penetrate into the wells. B27 medium was used for culturing. After pushing neurons into the wells, we waited at least 60 minutes to allow them to attach to the wells' wall. The pH was kept constant by super-flowing the medium with 10% carbon dioxide. After one hour the probes were transferred into the tissue culture incubator.

We estimated the probability of survival in the wells before transplantation the following way. In four probes, neurons were completely absent from the wells 24 hours after incubation, whereas in the remaining probe neurons were present in 2 of13 wells. We reason that the only way cells can quickly disappear is that they excape from the probe wells. A possible explanation for this is that the walls of the wells are not sufficiently covered with polylysine and thus there is not good enough terrain for the neurons to attach to.

Parallel culturing of the neurons from the same fetuses on polylysine coverslip glasses or Corning tissue culture dishes revealed good growth of neurons on these surfaces, As shown in the figure below.



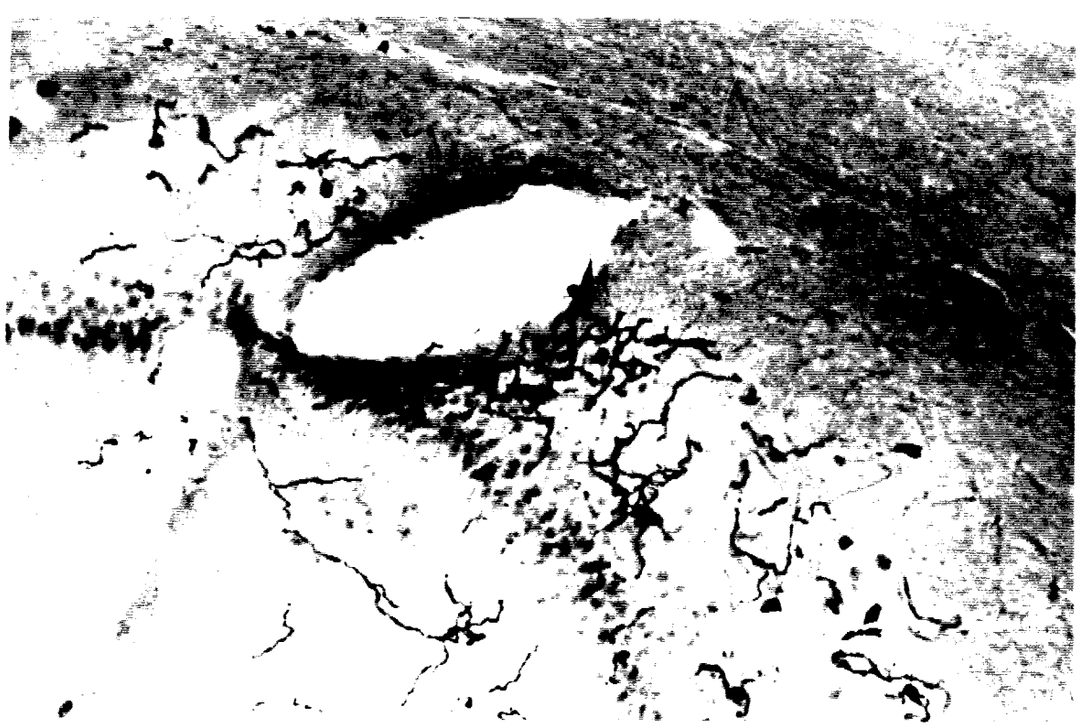
The cells in the photo were stained with the Molecular Probes Live/Dead dye kit, and we estimate that approximately 60-70% of the cells were green (alive) and 30-40% red (dead). Overall, we estimate that the probability of neuronal survival within the wells is approximately 0.5 in the best case, not counting the possible traumatic effects of moving the cells into wells.

We have noticed that some of the neurons stick to the surface of the silicone probe so hard that it was difficult to move them without strong pressure with the pusher. As a rule, we do not use such neurons for moving them into the wells because of the expected damage. Only neurons which can be moved easily are used. Additional trauma of the neurons can occur during preparation of the suspension.

Seventeen probes were transplanted into hippocampi of five rats with preliminary fimbria-fornix lesion. From one to three probes were implanted in

each hemisphere. The fimbria-fornix was removed by aspiration 1 to 4 weeks before probe grafting. After the probes have been inserted into the hippocampus, the handle area was broken and the opening was covered with paraffinized wax. The rats were perfused 1 to 2 months after the probe placement. Brains were cut in the coronal plane by a vibrotome. Initially, 200 to 400 μm sections were cut to make sure that the section contained a large part of the probe. After photographing the thick sections, they were cut again on a microtome to obtain 60 μm sections. These sections were then stained to reveal AChE-positive fibers.

In one rat, we found clear evidence of AChE-positive fiber outgrowth from surviving neurons in the wells. In this animal two out of six probes had a clearly identifiable network of AChE-positive axons in their vicinity. Below are photographs of two adjacent 60 micron sections showing stained axons from one probe. A higher magnification view of one of these sections is the top photo on the page following. The lower photo is a similar view of outgrowth from a second probe in the same rat, where the probe track is shown but the probe was lost in sectioning.



In the best case AChE-positive fibers were present several hundred microns septally and caudally to the probe site but not in other sections. Clearly, the sphere of the AChE-reactive fibers surrounded the probe placement. In another rat AChE-positive fibers were observed around the electrode but the fibers in this case could not be clearly traced back to the probe-tissue interface, mostly due to faint (unsuccessful) staining in these sections.

Although our success rate was very low, the findings in at least one of the five rats convincingly demonstrate that septal cholinergic cells can survive in wells and, more importantly, can outgrow in the host environment. The low success rate, on the other hand, is very disappointing. Large numbers of the cells are lost from the wells, and the expected survival of the ones in the wells do not exceed 50% in our hands. In the next quarter, the rest of our animals with 2 to 4 months survival time will be evaluated. In addition, we will try to improve adhesion of cells in the wells and also try placing more than one neuron in each well to increase the chance of survival as well as to keep the neurons in the wells. In every case, we will examine the proportion of the cells in the wells before transplantation.